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Polyphenolic profile and pharmacological activities of whips horse (*Luehea divaricata)* bark extracts studied using *in vitro* and *in vivo* systems

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ABSTRACT

Leaves and barks of *Luehea divaricata* (Malvaceae) are used in folk medicine for the treatment of infectious diseases, tumors, rheumatism and diabetes. This work presents *in vivo* experiments (rats and mice) supported by *in vitro* and chemical analyses in order to address the above mentioned folk medicinal uses. The phenolic constituents of both aqueous and hydroalcoholic extracts are mainly catechin derivatives (135.4 and 215.9 mg/g, respectively). Both extracts displayed strong antioxidant activity. They also showed antiproliferative activities against tumor cells, nitric oxide synthesis inhibition (indicative of anti-inflammatory activity) and antimicrobial activity against a variety of infectious agents. Paracetamol-induced liver injury in rats, which has a high inflammatory component, was substantially attenuated in animals treated with the hydro-alcoholic extract (200 mg kg^{-1} day^{-1}). This was deduced from aspartate aminotransferase and alanine aminotransferase measurements in plasma as well as from the hepatic activities of catalase and superoxide dismutase. Both extracts inhibited the pancreatic α-amylase with IC_{50} values of 11.50 ± 0.05 and 18.70 ± 3.87 μg/mL for the hydro-alcoholic and aqueous extract, respectively. Both extracts inhibited starch digestion in mice at the doses of 200 and 400 mg/kg. In general terms the results confirm several of the *vox populi* notions about the therapeutic potential of *L. divaricata,* the antidiabetic action, combined with the hepatoprotective effect, being the most relevant observations. Not less important is the economical perspective for the rural producers which may eventually increase their revenue by selling increasingly valued raw materials derived from *L. divaricata* based on its therapeutic properties.

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1. Introduction

Luehea divaricata Martius is a large tree belonging to the Malvaceae family, popularly known in Brazil as "açoita-cavalo", what literally translated means "whips horse". The plant is found mainly in the South and Southeast regions of Brazil, Paraguay and Argentina (Carvalho, 2008). The tree is very common in many small rural properties over the Brazilian territory and could eventually be economically explored as a source of medicinal preparations as its leaves and barks are traditionally used in folk medicine for the treatment of a series of infectious diseases (dysentery, leucorrhea), rheumatism, tumors, diabetes and weight loosing purposes (Dickel et al., 2007; Calixto-Júnior et al., 2016).

The widespread use of *L. divaricata* barks and leaves infusions in folk medicine has demanded a series of investigations about their biological activities and chemical compositions, which have in part substantiated the traditional folk knowledge. Antifungal properties, for example, have been attributed to a dichloromethane extract of *L. divaricata* barks (25 μg/filter disk), which was said to inhibit polymer synthesis or the cell wall assembly of *Neurospora crassa* (Zacchino et al., 1998). Also, anti-inflammatory, analgesic and immunostimulatory properties, which are suposedly behind the alleged anti-rheumatic effects, have been ascribed to a *L. divaricata* bark hydro-alcoholic extract. Epicatechin, stigmasterol, lupeol and α, β-amyrin were identified in this extract (Rosa et al., 2014). Additionally, tannins, flavonoids, such as quercetin and kaempferol, and saponins have already been identified in the leaves and bark of *L. divaricata*. (Vargas et al., 1991). The presence of vitexin, maslinic acid and epicatechin has also been reported to occur in barks and leaves (Tanaka et al., 2005). Another phytochemical screening of *L. divaricata* leaves identified flavonoids, tannins, saponins, and mucilage besides smaller quantities of alkaloids, fixed oils, anthocyanidins, carotenoids, and polysaccharides (Calixto-Júnior et al., 2016). An aqueous extract prepared from *L divaricata* leaves exerted a fair antioxidant activity and induced analgesia in rats with neuropathic pain (Courtes et al., 2015; Kroth et al., 2020). And, in a recent study, a modulatory effect on oxidative stress markers in the spinal cord of rats with neuropathic pain was attributed to an aqueous extract of *L. divaricata* leaves (Kroth et al., 2021).

Polyphenols are the largest group of secondary metabolites in plants and have been considered to exert multiple biological effects (Mouho et al., 2018; Gülçin et al., 2020; Bello et al., 2021; Zheleva-Dimitrova et al., 2021). To our best knowledge, an advanced characterization of the phenolic composition of the bark of *L. divaricata* has not yet been done. What is also lacking is a more extensive investigation on the alleged therapeutic effects of *L. divaricata,* especially those related to metabolism.

Taking into account the knowledge gaps mentioned above, the aim of the present work was to determine the phytochemical profile of *L. divaricata* bark aqueous and hydro-alcoholic extracts in order to expand the current knowledge on the chemical composition of this material and to correlate, in a preliminary way at least, chemical constituents to the alleged folk medicinal effects. In the latter domain, experiments were done in order to bring evidence to light that may confirm or not the antimicrobial, antitumor, anti-inflammatory and antidiabetic actions of *Luehea divaricata.*

2. Material and methods

2.1. Materials

The following enzymes and chemicals were purchased from Sigma-Aldrich (St. Louis, USA): pancreatic α-amylase (porcine; Type VI-B; A3176), A1031), maltose (M5885), glucose (G8270), potato starch (85642), acarbose ($C_{25}H_{43}NO_{18}$, MW 645; A8980), (ABTS), 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 2,2-diphenyl-1-picrylhydrazyl (DPPH), lipopolysaccharide (LPS), dexamethasone, sulforhodamine B, ellipticin, and streptomycin. Bifonazole was from Srbolek, Belgrade, Serbia. Gold Analisa was purchased from Labtest Brazil. The HPLC grade reagents (99.9%) were purchased from Fisher Scientific (Lisbon, Portugal). The commercial kits for the ALT and AST assays were purchased from Gold Analisa Diagnostica Ltda (Belo Horizonte, Brazil). The tumor cell lines were purchased from the Leibniz-Institut DSMZ.

2.2. Animals

Male Swiss mice, weighing between 35 and 45 g, were used for measuring starch absorption and male Wistar rats, weighing between 200 and 250 g, for evaluating hepatoprotection. All animals (three of them) were housed in cages with free access to water and food (standard show diet). The present work was approved by the Ethics Committee on the Use of Animals in Experimentation (CEUA) of the State University of Maringá (protocol number 6414170919).

2.3. Plant material and preparation of crude extracts

The barks of *L. divaricata* were collected in 2019, in Marialva, Paraná, Brazil (geographic coordinates: 23° 28′ 55″ ºS, 51° 47′ 44″ °W). Identification and characterization of the plant was carried out using botanical criteria, and a voucher specimen (HUEM # 35510) was deposited at the HUEM (Herbário da Universidade Estadual de Maringá). This herbarium possesses 27,000 plant registers and is under the technical responsibility of Prof. Maria Auxiliadora Milaneze Gutierre of the Biology Department. The collected barks of *L. divaricata* were dried under ventilation in an air-flow chamber and pulverized. The powder was submitted to two types of extraction, one using water and another using 70% ethanol (in water) as illustrated by Fig. 1. The solvent solute/solvent ratio for all extractions was 1:20 (m/v). A rotary shaker (130 rpm) was used to shake the suspensions during a period of 2 h at room temperature. After shaking, the suspension was centrifuged at 3000*g* for 30 min. Elimination of ethanol was accomplished using a rotary evaporator. The resulting aqueous solution was lyophilized and stored at −20 °C until use.

Fig. 1. Schematic view of the preparation of the *L. divaricata* bark extracts.

2.4. Phenolic profile of L. divaricata bark extracts

Methanol/water solutions (80:20, *v/v*) of the *L. divaricata* bark extracts, at a final concentration of 10 mg/mL, were filtered before the chromatographic procedures. The analysis was performed on a HPLC system (Dionex Ultimate 3000 UPLC, Thermo Scientific, San Jose, CA, USA) according to operating conditions, including identification and quantification, that were previously described in detail (Bessada et al., 2016). The chromatographic separation of the compounds was performed using a Waters Spherisorb S3 ODS-2C18 (3 μm, 4.6 mm × 150 mm, Waters, Mil-ford, MA, USA) column thermostatted at 35 °C, and an isocratic gradient using 0.1% formic acid in water and acetonitrile as elution solvents. A 7-level calibration curve of (+)-catechin was used for quantification purposes: $y = 84950x - 23200$, $R^2 = 1$, limit of detection = 0.44 μg/mL and limit of quantification = 1.33 μg/mL. The results were given as mg per g of extract.

2.5. Antioxidant activity

The antioxidant potentials of the extracts were evaluated by three different methods: 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical reduction assay, 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) reduction ($ABTS^+$) and ferric ion reducing power (FRAP). All assays were done using standardized procedures (Gülçin, 2012, 2020; Corrêa et al., 2015; Koehnlein et al., 2016). In the case of DPPH and $ABTS^+$ the outcomes were expressed as IC_{50} values (50% of free radical bleaching). The results of the FRAP assay were expressed in μmol TROLOX equivalents per mg extract.

2.6. Antimicrobial activity

Five percent dimethylsulfoxide (DMSO) solutions of the extracts were used for testing the antimicrobial activity. MIC (minimum extract concentrations with 100% inhibition of bacterial growth), MBC (minimum bactericidal concentration) and MFC (minimum fungicidal concentration) were evaluated as described elsewhere (Corrêa et al., 2015; Eruygur et al., 2019). The positive controls were streptomycin (bacteria) and bifonazole (fungi). The negative control was 5% DMSO.

2.7. Cytotoxicity analysis

The protocol previously described by Spréa et al. (2020) was used for assaying the cytotoxicity of the *L. devaricata* extracts against tumour cell lines. The positive control was ellipticin. For testing toxicity against a non-tumour cell line, primary porcine liver cells (PLP2) were obtained as described previously by Corrêa et al. (2015). Activities were given as GI_{50} values (μg/mL), i.e., the extract concentrations causing 50% inhibition of cell proliferation.

2.8. Anti-inflammatory activity

Inhibition of the production of nitric oxide (NO) in a lipopolysaccharide (LPS) stimulated murine macrophage cell line (RAW 264.7) was taken as an indicator for the anti-inflammatory activity of the *L. divaricata* extracts (Garcia-Aranda et al., 2020). The NO production was quantified using the Griess reagent, as described by Corrêa et al. (2015). Dexamethasone was the positive control and an incubation system free of LPS was taken as the negative control. The results were given as IC_{50} values (μg/mL), i.e., the concentration causing 50% inhibition of NO production.

2.9. Hepatoprotective activity

Hepatoprotective activity of the hydroalcoholic extract in rats was evaluated as described previously with some modifications (Soares et al., 2013a). To one group of healthy rats (n = 6) 200 mg/kg of the hydro-alcoholic *L. divaricata* extract were administered daily via the intragastric route during 14 days; to another group of healthy rats (n = 6), kept under exactly the same conditions, saline was administered (0.9% NaCl). After the 14 days had elapsed half of the animals in each of these two groups were injured by oral administration of 2 g/kg paracetamol (Soares et al., 2013a). The other half in each group received saline. After 48 h the animals were anesthetized with ketamine + xylazine (90 mg/kg + 9 mg/kg). After collecting blood samples, plasma for the hepatic marker

enzyme assays was obtained by centrifuging at 2500 rpm for 10 min. The livers were excised, weighed, frozen in liquid nitrogen and stored until use.

Commercial kits (Gold Analisa Diagnóstica, Belo Horizonte, Brazil) were used for assaying the aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities. The indicator enzymes in the AST and ALT assays were L-malate dehydrogenase and L-lactate dehydrogenase, respectively. The presence of one of these enzymes in the corresponding assay solutions allows to quantify the rates of oxaloacetate (AST) and pyruvate (ALT) productions using the oxidation of NADH as indicator. The spectro-photometric measurements were done at 340 nm (Bergmeyer, 1974). The results were expressed as international units per liter. One unit is the amount of enzyme that transforrms 1 μmol of substrate per minute.

For measuring the activities of catalase (CAT) and superoxide dismutase (SOD) the liver tissue was homogenized in a van Potter homogenizer with 10 vol of ice-cold 0.1 M potassium phosphate buffer (pH7.4). The homogenate was centrifuged at 9500 rpm for 15 min and the supernatant used for enzyme assay. The catalase activity was measured as described by Bergmeyer (1974). The superoxide dismutase (SOD) activity was assayed and scaled according to Marklund and Marklund (1974).

2.10. In vitro assay of alpha-amylase activity

In vitro inhibition of the pancreatic α-amylase by the *L. divaricata* extracts was evaluated as previously described with minor modifications (Kato et al., 2017; Gülçin et al., 2018). The temperature was 37 °C and the medium contained 20 mM phosphate buffer (pH 6.9) plus 6.7 mM NaCl. The substrate was potato starch. Enzyme and extract were pre-incubated for 15 min and the reaction was started by adding the substrate. The reaction time for measuring the initial rates was 10 min. The reducing sugars produced by starch hydrolysis were quantified according to Miller (1959). The standard was maltose and the optimized linear calibration curve was $y = a + bx = 0.0042 \pm 0.0055 + 0.097 \pm 0.0019x$ ($r = 0.998$), in which x is the maltose concentration (μmol/mL), y the absorbance at 540 nm, b the slope of the straight line and a the intercept on the y axis (not significant). Rates were calculated from the calibration factor (1/b), the reaction time (10 min), the corresponding dilution factor and expressed as μmol per minute.

2.11. Starch oral tolerance test

Starch absorption was evaluated by means of the starch tolerance test (Kato et al., 2017; Castilho et al., 2021). Mice were distributed randomly into nine groups (n = 3 mice per group) and underwent the following treatments: (1) group 1 received comercial corn starch intragastrically (1 g per kg of weight); (2) group 2 received 100 mg/kg of *L. divaricata* hydro-alcoholic extract plus corn starch (1 g per kg of weight); (3) group 3 received 200 mg/kg of *L. divaricata* hydro-alcoholic extract plus corn starch (1 g per kg of weight); (4) group 4 received 400 mg/kg of *L. divaricata* hydro-alcoholic extract plus corn starch (1 g per kg of weight); (5) group 5 received 100 mg/kg of *L. divaricata* aqueous extract plus corn starch (1 g per kg weight); (6) group 6 received 200 mg/kg of *L. divaricata* aqueous extract plus corn starch (1 g per kg weight); (7) group 7 received 400 mg/kg of *L. divaricata* aqueous extract plus corn starch (1 g per kg weight); (8) group 8 (negative control) received only tap water; (9) group 9 (positive control) received 50 mg/kg acarbose. Blood glucose was determined at times 0, 30, 60, 90 and 120 min after starch administration. Blood samples were collected from the tail vein and analyzed by means of a glucometer (AccuChek®).

2.12. Statistics

Differences between two means were assessed by Student's t-test. Data sets composed of more than two means were subjected to univariate variance analysis (ANOVA), with post-hoc Newman-Keuls-testing. Significance was accepted when $p \leq 0.05$.

3. Results and discussion

3.1. Polyphenolics in the extracts

Identification and quantification of the phenolic profile of the aqueous and hydro-alcoholic extracts of the *L. divaricata* bark are shown in Table 1 and an exemplificative phenolic profile is shown in Supplementary material (**SA1**). A total of 15 compounds were identified, all catechin derivatives, by comparison with descriptions in the literature (Peixoto et al., 2018; Dias et al., 2015). An exemplificative fragmentation pattern of the base unit of (−)-epicatechin is shown in Supplementary material (**SA2**). The peak 1 ($[M-H]^-$ at *m/z 451*), presenting a MS^2 fragment at *m/z* 289, was tentatively designated as an epicatechin-hexoside. Peak 2 ($[M-H]^-$ at *m/z* 739) corresponds to a procyanidin. Peaks 3, 7, and 9 ($[M-H]^-$ at *m/z* 865) present a MS^2 fragment at *m/z* 577 and are provisionally identified as catechin trimers. Peak 4 ($[M-H]^-$ at *m/z* 577) is probably a catechin dimer as it presented a MS^2 fragment at *m/z* 451. It can be tentatively considered a *β*-type (epi)catechin dimer. Peaks 5, 10, and 15 ($[M-H]^-$ at *m/z* 1441) are most probably *β*-type catechin pentamers considering that they presented a MS^2 fragment at *m/z 865*. The peak 6 ($[M–H]^-$ at *m/z* 289) was identified as (−)-epicatechin. Peaks 8, 11, and 14 ($[M–H]^-$ at *m/z* 1553), finally, were identified as β-type (epi)catechin tetramers.

Seven of the compounds found in the hydro-alcoholic extract were not detected in the aqueous extract. Hydro-alcoholic extraction is, thus, more efficient than aqueous extraction. Mainly for this reason, the phenolic content of the hydro-alcoholic extracts exceeds that of the aqueous extract by a factor of 1.595. Nonetheless, some particular compounds are present in higher amounts in the aqueous extract, notably the *β*-type (epi)catechin trimer (3.3 times more), *β*-type (epi) catechin pentamer (1.6 ×) and (−)-epicatechin (1.7 ×).

The phenolic profiles of the *L. divaricata* bark extracts are in agreement with those ones previously reported for a methanolic extract in terms of the abundance of flavonoids such as (−)-epicatechin (Tanaka et al., 2005). On the other hand, the bark seems to differ considerably from the leaves. An ethanolic extract of the leaves, for example, was shown to present phenolic acids and flavonoids derived from quercetin, kaempferol, rutin, vitexin and vicenin (Tirloni et al., 2018).

Table 1
Identification of the phenolic compounds of the *L. divaricata* stem bark. R_t means retention time and λ_{max} is the wavelength used for quantification. More details can be found in the Materials and methods sections.

Peak	R_t (min)	λ_{max} (nm)	[M-H]- (m/z)	MS² (m/z)	Tentative identification	Quantification (mg/g extract)	
						Aqueous extract	Hydro-alcoholic extract
1	4.86	278	451	289(100)	(Epi)catechin-hexoside	–	6.48 ± 0.32
2	5.29	280	739	587(100),577(43), 451(34),435(5),289(21)	Dimeric B-type C-glycosylated procyanidin	10.44 ± 0.36	9.93 ± 0.13
3	6.63	278	865	577(50),451(30),425(100),407(38),289(20)	β-Type (epi)catechin trimer	27.63 ± 0.38	8.44 ± 0.37
4	6.92	280	577	451(100), 575(40),425(5),407(5),289(5),287(10)	β-Type (epi)catechin dimer	–	33.28 ± 0.12
5	8.15	281	1441	865(100),287(54)	β-Type (epi) catechin pentamer	12.79 ± 0.8	7.88 ± 0.22
6	8.48	280	289	325(20), 289 (100), 245(100), 205(41)	(−)-Epicatechin	21.31 ± 0.32	12.63 ± 0.03
7	9.85	278	865	577(50),451(30),425(100),407(38),289(20)	β-Type(epi)catechin trimer	21.28 ± 0.34	20.83 ± 0.37
8	11.14	283	1153	865(68),713(20),695(2),577(22),575(40),425(5), 407(5),289(5),287(10)	β-Type (epi)catechin tetramer	–	19.36 ± 0.12
9	12.26	278	865	577(50),451(30),425(100),407(38),289(20)	β-Type(epi)catechin trimer	13.18 ± 0.38	12.34 ± 0.09
10	12.62	281	1441	865(100),287(54)	β-Type (epi) catechin pentamer	–	16.25 ± 0.17
11	13.20	283	1153	865(68),713(20),695(2),577(22),575(40),425(5), 407(5),289(5),287(10)	β-Type (epi)catechin tetramer	13.07 ± 0.13	13.7 ± 0.51
12	13.94	283	863	739(92),713(59),695(100),577(69),575(49),425 (14),407(10),289(6),287(12)	β-Type (epi)catechin trimer	–	14.87 ± 0.37
13	14.29	283	863	739(92),713(59),695(100),577(69),575(49),425 (14),407(10),289(6),287(12)	β-Type (epi)catechin trimer	–	11.94 ± 0.43
14	14.76	283	1153	865(68),713(20),695(2),577(22),575(40),425(5), 407(5),289(5),287(10)	β-Type (epi)catechin tetramer	15.66 ± 0.07	13.5 ± 0.19
15	16.30	281	1441	865(100),287(54)	β-Type (epi) catechin pentamer	–	14.56 ± 0.04
					Total phenolic compounds	**135.37 ± 1.89**	**215.97 ± 0.12**

3.2. Antioxidant activity

The high content in phenolic compounds suggests that both extracts might have antioxidant activity (Araújo et al., 2017; Musial et al., 2020). Three different methods were employed in the evaluation of the antioxidant activity of both extracts, namely the ABTS, DPPH and FRAP assays (Garcia et al., 2019). The results are shown in Table 2 and they reveal that in the three assays the hydro-alcoholic extract proved to possess a superior antioxidant capacity. For the DPPH and ABTS assays the IC_{50} hydro-alcoholic/aqueous extract ratios were, respectively 1.46 and 1.73. In the case of the FRAP assay the superiority of the hydro-alcoholic extract is given by a factor of 1.77.

The antioxidant activity found in the present work for the bark extracts seems to be more pronounced than that reported for leaf extracts of the same species. Bleaching of the DPPH radical by a methanolic extract of the *L. divaricata* leaves, for example, occurred with an IC_{50} of approximately 50 μg/mL (Arantes et al., 2014), meaning that in this respct the leaf extract is 3.75 times less effective than the hydro-alcoholic bark extract. An aqueous extract of the leaves was even less potent in bleaching DPPH as it presented a IC_{50} of 150 μg/mL (Kroth et al., 2020), which reveals an antioxidant capability that is 7.78 times less pronounced than that of the aqueous extract of the bark. It is interesting to note that the leaves of the related species *Luehea speciosa* revealed an IC_{50} of 107.74 μg/mL for DPPH bleaching (Port's et al., 2013). In principle, thus, if one considers the similarity of the above-mentioned extractions and the extractions of the present work, it can be concluded that the bark of *L. divaricata* contains a higher extractable antioxidant activity when compared to the leaves.

3.3. Antimicrobial activity

Results of the experiments in which the antibacterial and antifungal activities were evaluated are shown in Table 3. The antibacterial activities against three Gram-negative bacteria (*Escherichia coli*, *Salmonella* Typhimurium and *Enterobacter cloacae*) and three Gram-positive bacteria (*Staphylococcus aureus*, *Bacillus cereus* and *Listeria monocytogenes)* were measured. The minimal inhibitory concentrations (MIC) of the extract's values indicate that the hydro-alcoholic extract was more active against *B. cereus* and *E. coli.* For the other bacteria the MICs were not different, the same being valid for the MBC (minimal bactericidal concentrations) values.

The antifungal activity was evaluated against *Penicillium funiculosum, Penicillium verrucosum, Aspergillus fumigatus, Aspergillus versicolor, Aspergillus niger,* and *Trichoderma viride*. The MIC and MFC values presented in Table 3 indicate that the aqueous extract was

Table 2
Antioxidant activities of the *L. divaricata* extracts according to three chemical assays.

	DPPH ($IC_{50;}$ μg/mL)	ABTS ($IC_{50;}$ μg/mL)	FRAP (μM Trolox eq/μg)
Aqueous extract	19.27 ± 0.8^a	12.65 ± 0.6^a	747.96 ± 18^a
Hydro-alcoholic extract	13.31 ± 0.6^b	7.30 ± 0.2^b	1330.36 ± 47^b

The different superscripts in each row denote statistical differences at the 5% level.

Table 3
Antibacterial and antifungal activities of *L. divaricata* extracts. MIC, minimal inhibitory concentrations (mg/mL); MBC, minimal bactericidal concentrations (mg/mL); MFC, minimal fungicidal concentrations (mg/mL).

	Aqueous extract		Hydro-alcoholic Extract		Streptomycin		Bifonazole	
Antibacterial activity (mg/mL)	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MFC
Staphylococcus aureus (ATCC 6538)	3.0	6.0	3.0	6.0	0.1	0.2	–	–
Bacillus cereus (clinical isolate)	3.0	6.0	1.5	3.0	0.025	0.05	–	–
Listeria monocytogenes (NCTC 7973)	1.5	3.0	1.5	3.0	0.15	0.3	–	–
Escherichia coli (ATCC 35210)	3.0	6.0	1.5	3.0	0.1	0.2	–	–
Salmonella Typhimurium (ATCC 13311)	3.0	6.0	3.0	6.0	0.1	0.2	–	–
Enterobacter cloacae (ATCC 35030)	3.0	6.0	3.0	6.0	0.025	0.05	–	–
Antifungal activity (mg/mL)	**MIC**	**MFC**	**MIC**	**MFC**				
Aspergillus fumigatus (ATCC 1022)	0.8	1.5	0.8	1.5	–	–	0.15	0.2
Aspergillus niger (ATCC 1022)	0.8	1.5	0.8	1.5	–	–	0.15	0.2
Aspergillus versicolor(ATCC 11730)	0.8	1.5	0.8	1.5	–	–	0.1	0.2
Penicillium funiculosum (ATCC 36839)	0.4	0.7	0.8	1.5	–	–	0.2	0.25
Penicillium verrucosum I (isolated from food)	0.4	0.7	0.8	1.5	–	–	0.1	0.2
Trichoderma viride (IAM 5061)	0.8	1.5	1.5	3.0	–	–	0.15	0.2

more active against *Penicillium verrucosum*, *Penicillium funiculosum* and *Trichoderma viride* when compared to the hydro-alcoholic extract.

The antibacterial activity of *L. divaricata* has already been reported, namely against *Staphylococcus aureus* (Tanaka et al., 2005) and *Micrococcus luteus* (Coelho de Souza et al., 2004). Concerning the antifungal activity, Tanaka et al. (2005) found that a methanolic extract of the stem and its aqueous and ethyl acetate fractions were active against *Staphylococcus aureus* with a MIC value of 250 μg/mL. This MIC value is considerably lower than all values obtained in the present work with both aqueous and hydro-alcoholic extracts. The ample antibacterial action demonstrated in the present work possibly justifies the traditional and popular use of *L. divaricata* in the treatment of dysentery and other infectious diseases (Carvalho, 2008). With respect to these antimicrobial activities it is worth to mention that several catechins have been reported to be effective against viral infections and a recent study emphasizes the inhibitory role of the catechins against SARS-CoV-2 (Mhatre et al., 2021; Tran, 2013).

3.4. Cytotoxicity and potential anti-inflammatory activity

The results of the cytotoxicity and anti-inflammatory activity measurements are shown in Table 4. Both extracts inhibited the proliferation of the four cell lines. The most sensitive to both extracts were the HeLa and HepG2 cells, the inhibitory activity of the aqueous extract exceeding that of the hydro-hydroalcoholic extract by a factor of 1.43. The opposite occurred with the HeLa cells whereas the other two cell lines (NCI–H460 and MCF-7) were equally sensitive to both extracts. Hepatotoxicity was tested against porcine primary liver cells (PLP2) and the results are also shown in Table 4. The results reveal that both extracts were equally active in inhibiting proliferation of primary liver cells.

The antiproliferative activity against tumor cells found in the present work for *L. divaricata* bark extracts is similar to that one reported by Silva et al. (2012) for *Luehea candicans*. In the present study, however, the analyses were extended to non-tumor cells, more precisely to porcine primary liver cells (PLP2). In these cells the antiproliferative activity of both extracts, actually cell toxicity, was somewhat less pronounced than the similar activity on tumor cells, but still within a concentration range that might lead to concerns about their safety in mammals. In opposition to these concerns one must consider, however, the results of experiments in which signs for hepatotoxicity were searched by administrating ethanolic (Tirloni et al., 2018) and aqueous extracts (Kroth et al., 2020) of the leaves of *L. divaricata* to rats. No alterations in the plasma activities of AST and ALT in consequence of these treatments were found,

Table 4
Anti-tumor, hepatotoxic and potential anti-inflammatory activities of L. divaricata extracts. The results are the means ± standard deviations. Same letters for a given cell line indicate absence of a statistically significant difference between the corresponding pairs of values according to Students t-test ($p \geq 0.05$).

Assay	Aqueous extract	Hydro-alcoholic extract
Anti-tumor activity (GI_{50}, μg/mL)		
HeLa (cervical carcinoma)	63.48 ± 2.7[a]	45.51 ± 1.2[b]
NCI – H460 (non-small cell lung cancer)	57.11 ± 4.5[a]	57.51 ± 2.6[a]
MCF-7 (breast adenocarcinoma)	52.85 ± 3.4[a]	54.04 ± 3.2[a]
HepG2(hepatocellular carcinoma)	22.86 ± 5.3[a]	32.70 ± 0.8[b]
Hepatotoxic activity (GI_{50}, μg/mL)		
PLP2 (primary porcine liver cells)	83.22 ± 2.6[a]	83.02 ± 4.7[a]
Anti-inflammatory activity (IC_{50}, μg/mL)		
RAW 264,7 (LPS stimulated murine macrophages)	68.75 ± 3.8[a]	57.80 ± 4.9[b]

however, what disproves hepatotoxicity for the leaf extracts at the doses that were given. The treatment doses in these studies ranged from 100 to 1000 mg/kg and the treatment periods from 10 to 35 days.

Inhibition of the production of the pro-inflammatory mediator nitric oxide (NO) can be regarded as an indicator of potential anti-inflammatory action (Garcia-Aranda et al., 2020). In the search for a possible anti-inflammatory activity, thus, the action of both extracts on the production of NO by murine macrophages was measured. As revealed by Table 4 both extracts were able to inhibit NO production. The hydro-alcoholic extract was slightly more effective (by a factor of 1.19).

3.5. Hepatoprotective activity

Taking into account the antioxidant (Table 2) and anti-inflammatory activities of the *L. divaricata* bark extracts (Table 4) a hepato-protective action similar to that of fungal extracts, which also exert both activities (Soares et al., 2013a, 2013b), can be expected. For this reason, experiments similar to those reported by Soares et al. (2013a) were done in which hepatic injury was induced by the administration of high doses of paracetamol, as described in the Materials and methods section. Four parameters were measured: levels of alanine amino transferase (ALT) and aspartate amino transferase (AST) in the plasma and activities of catalase (CAT) and superoxide dismutase (SOD) in the liver. After 14 days of daily treatment with 200 mg/kg of the hydro-alcoholic extract, randomly selected treated and non-treated rats were submitted to paracetamol injury. The results of the whole series of experiments are shown in Fig. 2. The plasma levels of AST and ALT (panels A and B) in control rats (not injured) were low, as expected for healthy animals. The administration of the hydro-alcoholic extract caused no modifications. Paracetamol injury, on the other hand, caused a very pronounced increase in the plasma levels of both AST and ALT. Both aminotransferases are highly concentrated in the liver and an increase of these enzymes in the plasma is always strong indication of liver injury (Giannini et al., 2005). The increases in the AST and ALT levels in the plasma were partially prevented by the previous treatment with the hydro-alcoholic extract with 50% and 57% reductions, respectively. These results, evidently, can be interpreted as a hepato-protective effect, which can be brought about by several mechanisms including the anti-inflammatory and antioxidant activities.

The modifications in the hepatic activities of SOD and CAT, enzymes involved in the cellular antioxidant protection, are shown in panels C and D of Fig. 2, respectively. The paracetamol injury caused a 43% diminution in the hepatic SOD activity. The treatment with the hydro-alcoholic extract fully prevented this decrease and caused no modifications in rats that were not injured with paracetamol. The hepatic CAT activity was also substantially decreased by the paracetamol injury (66% diminution). In treated rats, however, this decrease was much less pronounced as it remained only 26% below the normal level. These actions on the activities of two key enzymes involved in the antioxidant protection in the liver can be regarded as an *in vivo* confirmation of the *in vitro* antioxidant actions of the *E. divaricata* bark extracts shown in Table 2. A similar action was also observed when rats with chronic constriction injury of the sciatic nerve were treated with an aqueous extract of the *L. divaricata* leaves (Kroth et al., 2020).

It is worth to emphasize that our experiments with the hydro-alcoholic bark extracts fully corroborate and complement previous observations that leave extracts of *L. divaricata* do not affect the levels of AST and ALT in healthy animals (compare non-treated and

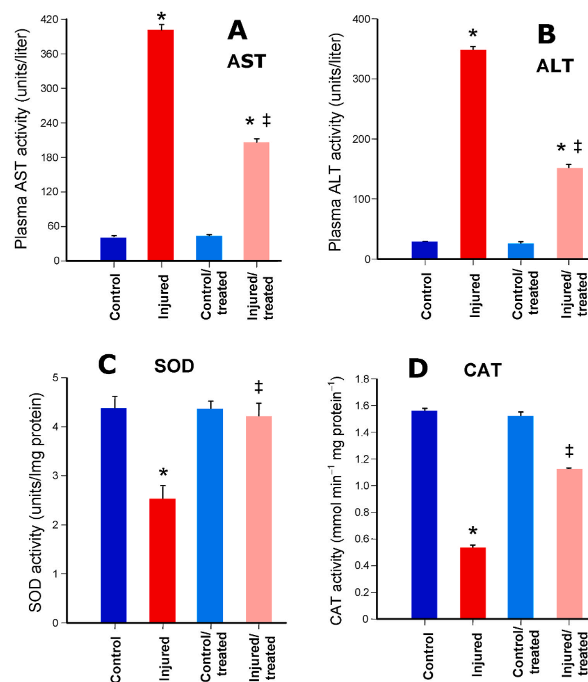

Fig. 2. Hepatoprotective effects of the hydro-alcoholic *L. divaricata* bark extract in rats. Values in the graphs are given as means ± standard errors of three determinations. Univariate variance analysis was applied followed by Student-Newman-Keuls post-hoc testing ($p < 0.05$). Asterisks (*) indicate values (injured or injured/treated) that are significantly different from the controls; the symbol (‡) indicates significant differences between the injured and injured/treated groups.

treated controls in Fig. 2; Tirloni et al., 2018; Kroth et al., 2020). This would also mean that *L. divaricata* hepatotoxicity does not manifest in the living rat at least not at the dose that was administered. A possible explanation is that the daily administration of 200 mg/kg for 14 days does not generate the plasma concentrations of the active molecules that are needed for a significant cytotoxicity, as indicated by the results in Table 4.

The hepatoprotective effect is a highly promising observation if one considers the fact that liver damage is a widespread disease which is characterized by a progressive evolution from steatosis to chronic hepatitis, fibrosis, cirrhosis and eventually hepatocellular carcinoma (Navarro and Senior, 2006; Soares et al., 2013b). It can also result from long term alcohol ingestion and chronic use of medications in which the regenerative capacity of the liver becomes dysfunctional (Saha et al., 2019). There is presently an intense search for effective herbal drugs which could be used as hepato-protective agents. This occurs because several of the currently employed synthetic drugs for liver diseases present unwanted side effects (Saha et al., 2019). Many of the natural products that have been proposed as hepato-protective agents, on the other hand, are rich in phenolic compounds. In this context, the hydro-alcoholic *L. divaricata* bark extract certainly presents favorable perspectives at least as an adjuvant for preventing and treating liver damage. Phenolic compounds could be acting as antioxidant and anti-inflammatory agents, but there is also steady accumulating evidence that favours an action of this class of compounds on gene regulation and expression and on protein modulation (Saha et al., 2019). The richness of the *L. divaricata* extracts in epicatechin derivatives is perhaps significant with respect to its hepato-protective effects. This is indicated by the finding that (−)epichatechin improves several serum markers in rats with in D-galactosamine-induced hepatitis (e.g., AST and ALT), along with a significant decrease in tissue damage (Shanmugam et al., 2017). It is clear thus, that further investigations in this respect with *L. divaricata* extracts are highly desirable. The daily dose that was administered to rats in our study was 200 mg/kg. For humans, however, the application of a well-known dose translation formula (Reagan-Shaw et al., 2007), makes one to expect that a 32.4 mg/kg dose should produce the same effects. However, it is worth to find out if treatments with lower doses are similarly effective.

3.6. *In vitro and in vivo* α-amylase inhibition

Several polyphenolics or extracts of various origins rich in polyphenolics have been demonstrated to inhibit α-amylases (Desseaux et al., 2018; Castilho et al., 2021). Considering the richness in polyphenolics of the extracts of the *L. divaricata* bark, inhibition of α-amylase is likely to occur. For this reason, the actions of both extracts on the activity of the porcine pancreatic α-amylase were measured. The results are shown in Fig. 3. The graph reveals that both extracts inhibited the α-amylase with a well-defined concentration dependence. Numerical interpolation revealed IC_{50} values of 11.50 ± 0.05 and 18.70 ± 3.87 μg/mL for the hydro-alcoholic and the aqueous extract, respectively. The hydro-alcoholic extract can be considered a somewhat a stronger inhibitor. However, inhibition approached 90% with both extracts as the concentrations were progressively raised to the maximal concentration used in this study (150 μg/mL). Expression of the IC_{50} values in terms of the total phenolic contents of each extract results in 2.48 μg/mL and 2.55 μg/mL for the hydro-alcoholic and aqueous extract, respectively.

Inhibitors of the pancreatic α-amylase are expected to reduce starch absorption (Kato et al., 2017; Castilho et al., 2021) if they also exert this effect *in vivo*. This possibility could be the reason behind the folk-medicinal use of *L. divaricata* bark extracts as antidiabetic agents (Calixto-Júnior et al., 2016) and even as weight loosing preparations (Dickel et al., 2007). This is a sound hypothesis if one takes into account that acarbose, a strong inhibitor of α-amylases, also inhibits starch absorption in both animals and humans (Saneusanio and Compagnucci, 1994). This is the reason why this compound, which is a trisaccharide, has been used in diabetes control for several decades. In order to find a possible parallel between acarbose and the *L. divaricata* bark extracts, the above mentioned hypothesis was tested in the present work by means of starch tolerance tests in mice. The results of this series of experiments are shown in Fig. 4. An oral starch load is normally followed by a transient increase in the blood glucose levels. This is confirmed by the experiments shown in Fig. 4 (panels A_1 and B_1). Pure water administration caused no alterations in blood glucose and allowed to de-

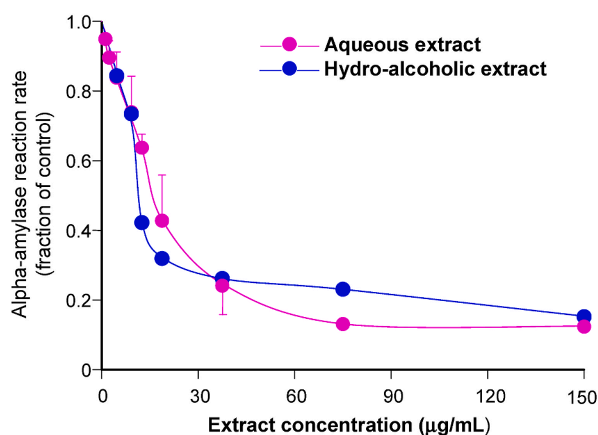

Fig. 3. Concentration dependence of the inhibition caused by the *L. divaricata* bark extracts on the pancreatic porcine α-amylase. Each datum point is the mean of three determinations. Standard errors of the mean cannot be seen when they are all smaller than the symbols. Reaction rates (v) were represented versus the extract concentration.

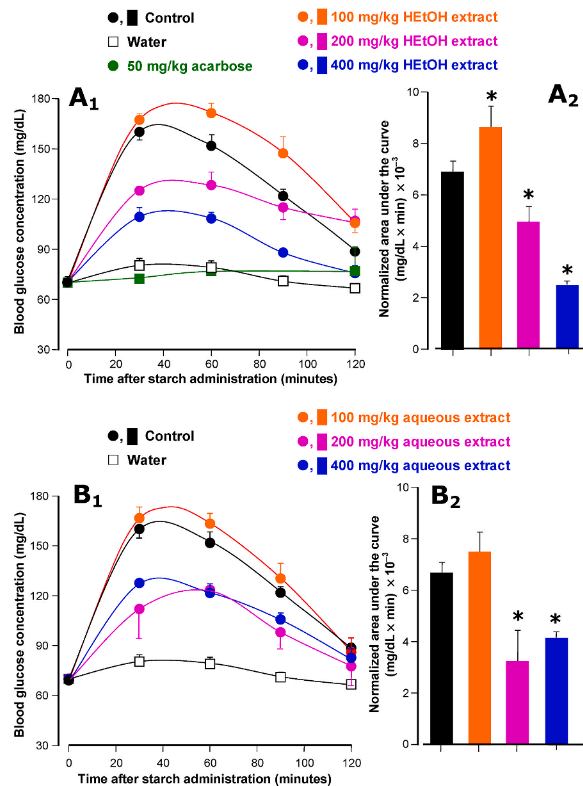

Fig. 4. Blood glucose concentration profiles after intragastric starch loads in mice and the effects of the hydro-alcoholic (panels A_1 and A_2) and aqueous (panels B_1 and B_2) *L. divaricata* bark extracts. The oral administration of commercial starch (1 g per kg body weight) was done immediately after the administration of the extracts or acarbose. The doses are given on the graphs. Plasma glucose was measured as described in Materials and methods. In panels A_1 and B_1 each value represents the mean ± mean standard error of 3–9 mice. Panels A_2 and B_2 show the areas under the curves obtained after the various treatments with the hydro-alcoholic or aqueous *L. divaricata* bark extracts, respectively, subtracted from the area under the curve obtained after water administration. Asterisks indicate statistical significance relative to the control curve ($p \leq 0.05$).

fine an appropriate baseline. The positive control experiment, which consisted in acarbose administration previously to the starch load (panel A_1), resulted in a strongly diminished response to starch administration. The hydro-alcoholic extract was administered at the doses of 100, 200 and 400 mg/kg, as revealed by panel A1 in Fig. 4. These loads are equivalent to the administration of 21.6, 43.1 and 86.4 mg/kg of polyphenolics, as can be calculated based in the data in Table 1. Surprisingly, the dose of 100 mg/kg produced a small increase in the glycemic levels along the whole experimental course. The doses of 200 and 400, however, were clearly inhibitory. With both doses the increases in blood glucose were substantially reduced in the first three half-hours. Convergence toward the basal level occurred only at the end of the experimental period. Panel A2 in Fig. 3 shows the areas between the experimental curves when starch was administered with or without the hydro-alcoholic extract administration and the curve obtained after water administration. This area has been considered a measure for the extensiveness of starch absorption. Statistical analysis reveals that all doses had a signficant effect, a small stimulation at the 100 mg/kg dose and progressive inhibitions with the 200 and 400 mg/kg doses. Numerical interpolation for obtaining the IC_{50} value revealed that 50% inhibition can be expected at the dose of 340 mg/kg (73.4 mg phenolics per kg).

The results of the experiments that were done with the aqueous extract are shown in panels B_1 and B_2 in Fig. 4. Doses of 100, 200 and 400 mg/kg were given, which correspond to 13.5, 27.1 and 54.1 mg polyphenolics per kg, respectively. With this preparation the stimulation at the 100 mg/kg dose presented a mere tendency, without statistical significance at the 5% level. Inhibition, however, took place with the 200 and 400 mg/kg doses with no clearly defined dose dependency, however.

Inhibition of the pancreatic α-amylase by *L. divaricata* extracts was not reported previously, but it can be expected based on the high content in catechins of both aqueous and hydro-alcoholic extracts (Hara and Honda, 1990; Desseaux et al., 2018). Actually, based on their IC_{50} values both extracts are relatively strong inhibitors of the pancreatic α-amylase. The hydro-alcoholic extract was a stronger inhibitor of the α-amylase than the aqueous extract as can be judged from the corresponding IC_{50} values. However, when the IC_{50} values are expressed in terms of their phenolic contents, they are practically the same. This fact strongly suggests that the polyphenolics may be important inhibitors of the α-amylase in both *L. divaricata* extracts investigated in the present work. Extracts of natural products that are inhibitors of α-amylase (or α-glucosidase) present an ample range of inhibitory potencies, but most of them do not have IC_{50} values smaller than 20 μg/mL. Actually, IC_{50} values are generally above 50 μg/mL and for several extracts values around 500 and even 1000 μg/mL or higher have been reported (Tundis et al., 2010; Silva et al., 2014; Kato-Schwartz et al., 2020; Castilho et al., 2021).

Inhibition of starch absorption by both *L. divaricata* extracts can be considered a natural consequence of the α-amylase inhibitory activity, as already observed for other natural products and acarbose (Silva et al., 2014; Kato-Schwartz et al., 2020; Castilho et al., 2021). However, based solely on the inhibitory effects of both extracts on α-amylase, one would not expect the strong tendency toward stimulation of starch absorption that was found at the lowest doses (100 mg/kg) given to the mice. In the case of the hydro-alcoholic extract the effect was even statistically significant. Its mechanism, however, cannot be inferred from the data obtained in the present work. It should be recalled, however, that the hydro-alcoholic extract caused a much more consistent inhibition of starch absorption, a phenomenon that may be related to its stronger action on the α-amylase, as evidenced by the *in vitro* experiments. It is also important to emphasize that, to our knowledge, the results about starch absorption inhibition by the *L. divaricata* extracts are the first confirmation of the folk medicinal notion about an anti-diabetic action of these preparations (Calixto-Júnior et al., 2016). It could equally be regarded as a preliminary evidence about the reported popular notion that extracts of *L. divaricata* can act as weight loosing agents, as reported earlier (Dickel et al., 2007) since inhibition of starch absorption is likely to reduce the energetic value of the ingested starchy foods in the human organism. Finally, although the doses of the *L. divaricata* bark extracts that are needed to inhibit starch absorption are higher than the effective doses of acarbose, it must be mentioned that the latter is a pure substance and the extract is a crude preparation, which is much easier to produce even by using rudimentary equipment that is usually available in the realm of folk-medicine.

4. Conclusions

In general terms the results confirm several of the *vox populi* claims about the medicinal potential of the *L. divaricata* stem bark extracts as anti-inflammatory, antimicrobial and antidiabetic agents. Evidence on a possible anti-tumour action is less clear: although anti-proliferative effects on tumor cells were found, these occur *in vitro* at concentrations that are close to the range that inhibits the proliferation of hepatic non-tumour cells. In vivo, anyway, hepato-protective effects, which have significant anti-inflammatory and antioxidant components, seem to predominate at doses of up to 200 mg/kg. As a final conclusion it can be said that the hepato-protective action, combined with the antidiabetic effects in consequence of the inhibition of starch digestion, can perhaps be regarded as the most significant observations of the present work. Not less significant, though at a different domain, is the fact that the active extract preparation was obtained from the bark of a tree, a material that is usually discarded. This is a feature that could eventually contribute substantially for economic development and sustainable production, besides representing a possibility of increasing revenue due to value aggregation to otherwise low-valued products.

Author contributions

Jéssica A.A Garcia-Manieri: Conceptualization, Methodology, Formal analysis, Investigation, Writing - Original draft. **Vanesa Gesser Correa**: Methodology, Formal analysis, Investigation, Writing review & editing. **Rúbia Carvalho Gomes Corrêa**: Methodology, Formal analysis, Investigation. **Maria Inês Dias**: Methodology, Formal analysis, Investigation. **Ricardo C. Calhelha**: Methodology, Formal analysis, Investigation. **Marija Ivanov**: Methodology, Formal analysis, Investigation. Marina Soković: Writing review & editing. **Lillian Barros**: Writing review & editing. **Isabel C.F.R. Ferreira**: Writing review & editing. **Adelar Bracht**: Conceptualization, Formal analysis, Investigation, Writing review & editing. **Rosane Marina Peralta**: Conceptualization, Formal analysis, Investigation, Writing review & editing.

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Declaration of competing interest

The authors declare no conflict of interest.

Data availability

No data was used for the research described in the article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2022.102530.

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